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CONTROL OF CORPORA ALLATA FUNCTION IN LARVAE OF *GALLERIA MELLONELLA*

FRANTISEK SEHNAL AND NOELLE A. GRANGER

Institute of Entomology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia and Department of Developmental and Cell Biology, University of California, Irvine, California 92664

In most insects larval development is controlled by juvenile hormone, which is produced by the corpora allata (*CA*). When a threshold concentration of the hormone is reached before a certain time during the larval intermolt period, the next molt gives rise to another larva; but if this concentration is not reached, metamorphosis begins (Novák, 1966). In many species, metamorphosis starts after a determined number of larval instars, but in some species it may occur earlier or later depending on environmental conditions. This indicates that the function of the *CA* is controlled by both internal and external factors, but little is known of the mechanism of this control. In this study we examined the control of the *CA* in the larvae of the waxmoth, *Galleria mellonella* L.

Piepho (1940) showed that it was possible to prevent metamorphosis in *Galleria* by supplying last instar larvae with extra *CA*. The clearest results were obtained when the larva received three complexes of brain-corpora cardiaca-corpora allata (brain-*CC*-*CA*), but three pairs of *CC*-*CA* produced similar effects (*CC* cannot be surgically separated from *CA*). Since implantation of three brains into mature larvae had no effect, Piepho ascribed the action of the complexes to *CA* activity.

More recently it has been shown that implantation of brains into freshly ecdysed last instar larvae of *Galleria* induces development of supernumerary larvae (superlarvae) (Sehnal, 1966a; Pipa, 1971). Krishnakumaran (1972) further demonstrated that brains from injured larvae are capable of evoking development of larval-pupal intermediates when implanted into larvae during the second half of the last instar. Pipa (1971) suggested that the implanted brains caused the extra larval molt by stimulating secretion of ecdysone from the host's prothoracic glands, but Krishnakumaran (1972) assumed that the brains stimulate both the *CA* and the prothoracic glands. We have approached this problem experimentally and examined in detail the role of the brain in the control of the *CA*.

Materials and Methods

A laboratory strain of the waxmoth *Galleria mellonella* L. was maintained on an artificial diet at 30° C and 80% r.h. in total darkness (Sehnal, 1966b). Penultimate and last instar larvae of known age were used for the experiments. The following surgical procedures were employed:

Implantations

Most of the implants were parts of the cerebral neuroendocrine complex. The organs were dissected from last instar larvae in insect saline (Novák, 1966) and

after 5–40 minutes were implanted into water-anaesthetized host larvae. A V-shaped cut was made with iridectomy scissors in the dorsolateral region of the 5th abdominal segment of the host, and the implants were inserted into the body cavity by means of two fine glass rods.

Allatectomy and denervation of CA

The larva was anaesthetized in water for about 5 minutes, rinsed quickly in 70% ethanol and then in insect saline, and placed dorsal side down in a Petri dish lined with paraffin and filled with insect saline. The neck region of the larva was stretched by means of two pairs of pins which were fixed to the paraffin in the bottom of the dish. The integument of the neck region was cut with a sharp scalpel so as to expose the tips of the tentorium. The muscles near the posterior ends of the tentorium were pulled aside with a hooked needle and the *CC-CA* complex, which was then visible in the incision, was removed with a pair of fine forceps. Since the *CC* and the *CA* are closely attached to each other, it was impossible to remove the *CA* and leave the *CC* intact. The removed glands were either discarded or immediately replaced approximately in their original position. This latter procedure left the insect with denervated glands. Although we handled the glands as gently as possible, some of those inserted back into the body cavity were undoubtedly damaged, so that these insects were effectively allatectomized rather than denervated. In some cases another implant of either brain or *CC-CA* complex was inserted into the neck incision immediately after the removal or replacement of the glands.

Following the operation, the pins fixing the insect were removed, and the insect was blotted on absorbent paper and transferred to a Petri dish. The operated insects were left at room temperature for 24 hours before being supplied with food and returned to 30° C. Some of them received implants three days later, which were inserted into the abdomen in the manner described above.

Results

Inhibition of metamorphosis by various implants

Table I demonstrates that sham operated animals or animals implanted at the beginning of the last instar with three subesophageal ganglia, three pieces of gut, or three sets of thoracic ganglia underwent a normal larval-pupal transformation. The same results were also observed following implantations of different muscles or portions of fat body. The larval-pupal transformation of *Galleria* was suppressed, however, by the implantation of different parts of the cerebral neuroendocrine complex. Implantation of three entire brain-*CC-CA* complexes was the most effective, but all implants containing either brain or *CC-CA* evoked a similar effect. The majority of the insects supplied with these implants molted into morphologically perfect superlarvae (Sehnal and Schneiderman, 1973). Some of them continued to develop as larvae for yet another larval instar (second extra larval instar), and a few of those implanted with three entire complexes underwent as many as four extra larval molts.

TABLE I

Development of larvae supplied with different implants within 24 hours after the last larval-larval ecdysis

Implant†	Number of operated insects	Per cent of insects entering extra larval instars*				
		n + 1 instar	n + 2 instar	n + 3 instar	n + 4 instar	n + 5 instar
Brain	92	61%	17%	0	0	0
CC-CA	59	67%	17%	0	0	0
Brain-*CC-CA* complex	157	89%	74%	24%	9%	2%
Subesophageal ganglion	30	0	0	0	0	0
Thoracic ganglia	21	0	0	0	0	0
Gut	11	0	0	0	0	0
Sham operated	25	0	0	0	0	0

* The remaining insects pupated

† Tissue from three last instar larvae was implanted into each host larva.

Implantations performed at later periods of the last larval instar revealed significant differences in the action of the different parts of the cerebral neuroendocrine complex (Table II). Larvae responded to brains only when they were implanted within 48–60 hours after the last larval-larval ecdysis, but they responded to the implantation of glands as late as 120 hours after the ecdysis. Even when the number of implanted brains was increased to six, larvae older than 60 hours failed to respond. Insects supplied with either three brain-*CC-CA* complexes or with three pairs of *CC-CA* 60 hours or more after the last larval-larval ecdysis molted into various larval-pupal intermediates (Piepho, 1940; Sehnal and Schneiderman, 1973). While the formation of intermediates was common after the implantation of complexes, the implantation of *CC-CA* often led to a great prolongation of the instar, with the insects eventually pupating.

An important characteristic of the action of the implants was their effect on the length of the instar. For example, in experiments with freshly ecdysed last instar larval hosts, the resulting superlarvae ecdysed 108–120 hours after the implantation of brains or complexes, but 168 hours after the implantation of *CC-CA*. The unaffected insects ecdysed as pupae within 192–198 hours after the implantation of brains or complexes, but within 324 hours after the implantation of *CC-CA*. A few of the larvae implanted with *CC-CA* lived for more than two weeks and grew distinctly larger than normal last instar larvae before eventually pupating. This effect was never observed following brain implantations.

Effects of implantations into allatectomized larvae

The implanted glands could prevent metamorphosis by producing juvenile hormone. The brains, however, might act by eliciting precocious secretion of ecdysone from the prothoracic glands, as suggested by Pipa (1971). The high titer of ecdysone would then induce an accelerated molt before any metamorphic change could occur. On the other hand, the implanted brains could also stimulate the host *CA* to secrete juvenile hormone; the increased hormone titer would then

TABLE II

Development of larvae supplied at different times after the last larval-larval ecdysis with implants from 48–120 hr last instar larvae.

Hosts		Development to superlarvae		Development to intermediates		Development to pupae†	
Age (hrs)	Number	Per cent of total insects	Length of instar (hrs)*	Per cent of total insects	Length of instar (hrs)*	Per cent of total insects	Length of instar (hrs)*
Implantation of 3 brains							
12	89	54	120	0	—	45	192
48	9	44	151	0	—	56	180
60	11	9	192	0	—	91	204
72	25	0	—	0	—	100	192
120	33	0	—	0	—	100	198
Implantation of 3 pairs of glands (c. cardiaca-c. allata)							
12	73	74	168	0	—	17	324
48	23	70	159	0	—	27	281
60	20	35	163	10	204	40	254
120	16	6	240	6	264	63	254
Implantation of 3 complexes (brain-c. cardiaca-c. allata)							
12	36	89	108	0	—	11	198
60	64	48	173	24	170	17	214
120	40	0	—	70	238	30	204
Sham operated							
12	25	0	0	0	0	100	197
48	23	0	0	0	0	100	181
72	25	0	0	0	0	100	178
120	25	0	0	0	0	100	192

* The normal length of the last larval instar is 180–190 hours. The age of the host larvae at the time of implantation was ±8 hours.

† Percent of insects which died during the experiments may be calculated from the data listed in the table.

suppress metamorphosis. To distinguish between these two possibilities, brains were implanted into allactectomized larvae.

As shown in Table III, larvae allatectomized in the penultimate instar underwent a precocious metamorphosis. Precocious metamorphosis never occurred in insects from which one corpus cardiacum-corpus allatum complex was removed (half-allatectomy). However, in the last larval instar, neither complete allatectomy nor half-allatectomy had any significant effect on development. Allatectomy also did not alter the response of the larvae to the implantation of *CC-CA*. A majority of the allatectomized insects supplied with glands underwent an extra larval molt,

TABLE III

Development of allatectomized larvae supplied with different implants from 48–120 hr last instar larvae

Operation	Number of larvae*	Development to larvae and intermediates		Development to pupae		Dead‡
		% of survivors	Length of instar† (hr ± SD)	% of survivors	Length of instar† (hr ± SD)	
Host larvae allatectomized 24 hrs after the penultimate larval-larval ecdysis						
Allatectomy	12	0	—	100	not recorded	0
Half-allatectomy	12	100	not recorded	0	—	0
Host larvae allatectomized 12 hrs after the last larval-larval ecdysis						
Allatectomy	7	0	—	100	204 ± 24	2
Allatectomy plus implantation of 3 brains	42	0	—	100	214 ± 21	15
Allatectomy plus implantation of 6 brains	12	0	—	100	245 ± 21	3
Allatectomy plus implantation of 3 pr of glands	36	72	214 ± 48	28	432±201	18
Allatectomy plus implantation of 6 pr of glands	8	100	218 ± 80	0	—	2
Half-allatectomy plus implantation of 3 brains	25	39	142 ± 24	61	257 ± 37	2
Sham operated	21	0	—	100	284 ± 46	3
Host larvae allatectomized 72 hrs after the last larval-larval ecdysis						
Allatectomy plus implantation of 3 brains	10	0	—	100	250 ± 46	3
Allatectomy plus implantation of 3 pr of glands	9	50	415 ± 97	50	569±327	3
Sham operated	35	0	—	100	264 ± 77	6

* Only animals which survived more than 24 hrs after operation are included.

† The first 24 hours after the operation were not included in the instar length.

‡ This column includes animals which either died or did not moult during the three weeks of the experiments.

and the remainder pupated after a delay averaging 18 days. The delay was greater than that observed after the implantations of *CC-CA* into intact larvae and was particularly common in insects allatectomized 72 hours after the last larval-larval ecdysis. Some allatectomized larvae lived for 3–6 weeks after the implantation of *CC-CA* before eventually dying.

The allatectomized insects failed to respond to the implantation of brains, however, and molted into normal pupae. Even the implantation of six brains did not elicit extra larval development. In contrast, three brains were adequate

TABLE IV

Development of larvae with nerves innervating the corpora cardiaca-corpora allata severed and with implants supplied 0 or 72 hrs later

Operation	Number of larvae*	Development to larvae and intermediates		Development to pupae		Dead‡
		% of survivors	Length of instar† (hr ± SD)	% of survivors	Length of instar† (hr ± SD)	
Nerves servered 24 hrs after the penultimate larval-larval ecdysis						
Nerves severed	21	95	not recorded	5	not recorded	
Nerves severed 12 hrs after the last larval-larval ecdysis						
Nerves severed	45	7	156± 0	93	328±120	12
Nerves severed and 3 brains implanted	39	48	189± 96	52	278± 61	12
Nerves severed and 3 brains implanted 72 hr later	30	15	187± 36	85	288± 67	3
Sham operated	21	0	—	100	284± 46	3
Sham operated and 3 brains implanted	8	57	175± 12	43	252± 42	1
Nerves severed 72 hrs after the last larval-larval ecdysis						
Nerves severed	36	7	360±101	93	384±185	5
Nerves severed and 3 brains implanted	36	20	391±132	80	317± 98	1
Sham operated	35	0	—	100	264± 77	6
Sham operated and 3 brains implanted	6	0	—	100	308± 55	0
Nerves severed 120 hrs after the last larval-larval ecdysis						
Nerves severed	37	3	264± 0	95	449± 94	5
Nerves severed and 3 brains implanted 72 hr later	11	12	576± 0	88	357± 62	2
Sham operated	16	0	—	100	271± 14	1

* Only animals which survived for more than 24 hrs after the operation are included.

† The first 24 hrs after the operation were not counted.

‡ This column includes animals which either died or did not moult during the four weeks of the experiments.

to induce development of superlarvae in nearly 40% of half-allatectomized insects; the rest of the half-allatectomized larvae pupated with a slight prolongation in the length of the instar. These results clearly demonstrate that the presence of at least one corpus cardiacum-corpus allatum complex is essential for the effect of the implanted brains.

Development of larvae with denervated glands

Table IV demonstrates that freeing the host *CC-CA* of all nervous connections had no effect on the development of penultimate instar larvae. With the exception of a single larva which pupated (probably due to the damage of its *CC-CA* during the operation), all operated insects ecdysed into normal last instar larvae.

Severing the nerves to the *CC-CA* during the last larval instar had no effect on the larval-pupal transformation of 93–95% of the operated larvae, although metamorphosis occurred significantly later than in intact or allatectomized larvae (*cf.* Tables II and III). Suppression of metamorphosis did occur in a few cases. Those affected animals which had been operated 12 hours after the last larval-larval ecdysis produced perfect superlarvae, while those operated at 72 and 120 hours after the ecdysis molted, after about two weeks, into imperfect superlarvae and larval-pupal intermediates. In addition, nearly 10% of all operated insects failed to pupate for as long as four weeks after the operation.

Severing their nervous connections did not prevent the glands of freshly ecdysed last instar larvae (12 hours after the ecdysis) from responding to the implanted brains (Table IV). After the implantation of three brains, about half of these larvae developed into superlarvae; the occurrence of extra larval instars in these animals was thus only slightly less frequent than in intact larvae following the implantation of three brains (Table I). However, when the implantation of brains was postponed until 72 hours after the denervation of the glands, only 15% of the experimental larvae underwent an extra larval molt.

Most significantly, denervating the glands 72–120 hours after the last larval-larval ecdysis restored their sensitivity to the implanted brains. Although intact larvae of this age never respond to implanted brains, 10–20% of the denervated insects molted, after brain implantation, into imperfect superlarvae and larval-pupal intermediates.

Thus severing the nerves to the *CC-CA* complex during the last larval instar partially restores gland activity and also their sensitivity to implanted brains. Following denervation the glands secrete enough juvenile hormone to cause a significant prolongation of the instar and, in a few cases, the development of superlarvae. The low numbers of operated insects undergoing the extra larval development indicate that some inhibition of *CA* function persists after nerve severence. The source of the inhibition could be the brain. However, its inhibitory action appears to be conditioned by the brain's integrity with the rest of the nervous system. When the brain with attached *CC-CA* is transplanted into another larva, the glands resume hormone production and induce extra larval development in the host (*cf.*, Table II). In addition, it is clear that the role of the nervous connections from the subesophageal ganglion to the *CC-CA* complex in the inhibition of gland function has not been defined.

Discussion

Freshly ecdysed last instar larvae of *Galleria mellonella* respond to the implantation of three brains with an extra larval molt. Since this effect also occurs in larvae from which one *CA* has been removed, but not in larvae from which both *CA* have been removed, the implanted brains must exert their effect by activating the *CA* of the host. The activation is not mediated by the host nervous

system because brains are also active in larvae in which all nervous connections to the *CC-CA* are severed. In another paper we consider the nature of this blood-borne allatotropic factor (Granger and Sehnal, 1974).

A humoral, presumably neurohormonal, control of the *CA* by the brain has also been demonstrated in various adult insects (*cf.* Cassier, 1967; Engelmann, 1970) and also in some larvae. Dreschner (1960) proposed an allatotropic function for the brain in nymphs of *Periplaneta americana*. Girardie (1965, 1967) showed that neurosecretory cells in the brains of *Locusta migratoria cinerascens* nymphs produce both stimulatory and inhibitory factors affecting the *CA*, although the inhibitory factor may inactivate the released juvenile hormone rather than repress its production. Ozeki (1962) presented indirect evidence for a humoral control of the *CA* in the earwig *Anisolabis maritima*: inactive *CA* became activated when cultured in penultimate instar larvae for ten days. Similarly, Fukuda (1962) showed that transplanted *CA* in *Bombyx mori* often behave in a manner corresponding to the developmental stage of the host.

The sensitivity of *Galleria CA* to the allatotropic factor is maintained for the first 48–60 hours of the last larval instar, after which it seems to be lost. However, the insensitivity can be partly abolished by severing the nerves innervating the *CC-CA* complexes. In a few instances denervation itself, without brain implantation, activates the glands and suppresses metamorphosis. These results indicate that the secretory function of the *CA* is inhibited via the nerves between 48–60 hours after the last larval-larval ecdysis.

The possibility of nervous control of the *CA* was suggested by Scharrer (1946) and later by other authors, but no conclusive evidence for this control in larvae is available. The published data demonstrate that the integrity of the allatal nerves has a different significance for *CA* activity in different species. For example, in the cockroach *Leucophaea maderae*, severing the *CA* nerves has a strong stimulating influence on the glands, and all operated insects undergo an extra larval molt (Lüscher and Engelmann, 1960). In contrast, this operation stimulates extra larval development in only 7% of the cases in *Galleria* (present results) and never in *Bombyx mori* (Bounhiol, 1957). In *Anisolabis maritima*, severing the *CA* nerves causes a partial inhibition of *CA* activity (Ozeki, 1962), an effect opposite to that observed in *Leucophaea maderae*.

These and other data in the literature indicate that insect *CA* are controlled by both activating and inhibiting stimuli which reach the glands either via the haemolymph or via the nerves. The significance of these two mechanisms of transmission appears to be different in different species. In certain stages of development, the effect of one type of stimulus seems to persist, and the glands do not respond to the opposite stimulus. For example, *CA* dissected from *Anisolabis maritima* during the first three quarters of the last larval instar (Ozeki, 1965), or from *Bombyx mori* during the second half of the last larval instar (Fukuda, 1944), remain inactive when transplanted into allatectomized larvae of the penultimate instar; however, the internal milieu of penultimate instar larvae of either species normally has a stimulating influence on *CA* activity (Fukuda, 1962; Ozeki, 1962). Oshiki and Morohoshi (1973) have found that when a certain strain of *Bombyx mori* is exposed to temperature and moisture shocks after the third larval ecdysis, the *CA* become inactive during the fourth larval instar instead of during the fifth, and a premature metamorphosis ensues. When inactive glands from

shocked fourth (last) instar larvae are transplanted into allatectomized and untreated fourth instar larvae, the transplanted *CA* become activated and the host larvae undergo a normal fifth larval instar. It was further observed that activation of the *CA* occurs much less frequently when whole brain-*CC*-*CA* complexes are transplanted. In this case the nervous connections to the brain are obviously important for the maintenance of *CA* inhibition.

In summary, we conclude that the activity of the *CA* is determined by an interplay of activating and inhibiting stimuli of varying intensity, whose effects may persist for various lengths of time. Similar mechanisms seem to control the function of the *CA* in adult insects, as was recently demonstrated by Baehr (1973) in *Rhodnius prolixus*.

Studies on volume changes of the *CA* led Kaiser (1949) and Novák (1954) to conclude that the amount of juvenile hormone secreted during larval development depends primarily on the size of the glands. The authors assumed that the concentration of juvenile hormone becomes gradually smaller in succeeding larval instars because the *CA* grow more slowly than the rest of the body. As the result of a disproportion between *CA* volume and body size in a certain instar, the glands do not produce a sufficient concentration of juvenile hormone, and metamorphosis begins. The theory may be valid in considering the phylogenetic origin of insect metamorphosis, but does not seem to apply to certain present-day species. It was disproven in the case of *Leucophaea maderae* (Luscher and Engelmann, 1960) and it is also inconsistent with our data on *Galleria*. In our experiments, removal of one of the two *CA* from penultimate instar larvae undoubtedly caused a great disproportion in the ratio of gland volume to body size, but had no effect on the further development of the larvae. This indicates that more complex mechanisms than the allometric growth of the *CA* control the titer of juvenile hormone in the larva; the nature of some of these mechanisms was previously discussed.

Up to 48 hours after the last larval-larval ecdysis, a larva of *Galleria* may develop either into a perfect superlarva or into a normal pupa, depending on the circulating titer of juvenile hormone (Piepho, 1940; Sehnal and Schneiderman, 1973). Both internal and external stimuli determine which of these developmental possibilities will be realized. Although metamorphosis normally begins in the seventh larval instar, it may occur earlier or later if the insects develop in unfavorable conditions (Sehnal, 1966b).

We assume that after each larval-larval ecdysis, the brain, in response to different internal and external stimuli, is programmed either for larval development or for metamorphosis. When it is programmed for larval development, it releases the allatotropic factor and the *CA* begins secreting juvenile hormone in amounts sufficient to induce larval development. Secretion of the brain hormone, which stimulates the prothoracic glands, seems to be accelerated. On the other hand, when the brain is programmed for metamorphosis, the *CA* are inactivated via their nervous connections between 48 and 60 hours after the ecdysis. The glands *in situ* cannot thereafter be fully activated by the insect's own brain, at least until the end of the last larval instar. This persistent inactivation of the *CA* prevents lethal deviations from normal development, since if the glands resumed their full activity in the course of the instar, inviable larval-pupal intermediates would be produced. Hence, a double-control mechanism—lack of the

allatotropic factor and nervous inhibition of the *CA*—assures that the insect develops either into a perfect larva or into a perfect pupa.

The double-control mechanism may play yet another role, namely in governing the precise level of juvenile hormone secretion. Denervation of the *CC-CA*, as well as the implantation of the *CC-CA* into allatectomized larvae, often leads to a significant delay in the larval-pupal ecdysis, similar to that which occurs after administration of low doses of juvenile hormone and its analogues. This prolongation of the instar resembles the diapause which occurs in *Galleria* under certain environmental conditions (Sehnal, 1966b). A similar larval diapause in *Chilo supressalis* (Fukaya, 1962) and a profound larval diapause in *Diatraea grandiosella* (Yin and Chippendale, 1973) are caused by a low level of the juvenile hormone secretion. We assume that the same is true in *Galleria*. Since prolongation of the instar occurs after severance of the nerves to the *CC-CA* complex, the low level of hormone secretion by the *CA* may occur as a result of the partial removal of their nervous inhibition. In another paper we examine variations in the allatotropic activity of the brain during the penultimate and last larval instars of *Galleria* (Granger and Sehnal, 1974). The experiments of Krishnakumaran (1972) indicate that the allatotropic activity of the brain of *Galleria* may also vary in response to a stimulus such as injury. Insects may use this allatotropic control by their nervous system to alter and adjust the secretory activity of the *CA* during their entire development.

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Summary

1. The implantation of three brains into freshly ecdysed last instar larvae which possess at least one of the two corpora allata induces extra larval development. Implanted brains appear to produce a neurohumoral allatotropic factor.

2. Corpora allata seem to be inhibited via their nervous connections 48–60 hours after the last larval-larval ecdysis and become insensitive to the allatotropic factor. Severance of the nerves innervating the corpora allata and adjacent corpora cardiaca induces extra larval development in 7% of the experimental insects and restores sensitivity to the implanted brains in as many as 20%.

3. Severance of the nerves to the corpora cardiaca—corpora allata complexes or implantation of corpora cardiaca—corpora allata complexes into allatectomized larvae in some instances causes a considerable prolongation of the last larval instar. It is suggested that disconnection of the corpora allata from the brain partly removes their inhibition and induces secretion of a low titer of juvenile hormone which is then responsible for the delay in pupation.

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